EXHIBIT B - VACCINE CANDIDATE REPORT

TULANE UNIVERSITY PRIMATE RESEARCH CENTER
&
SMITHKLINE BEECHAM BIOLOGICALS

COLLABORATIVE RESEARCH PROGRAM

REPORT ON THE IDENTIFICATION OF A NEW ANTIGEN OF *BORRELIA GARINII* THAT IS A VACCINE CANDIDATE

MARIO T. PHILIPP, PH. D.

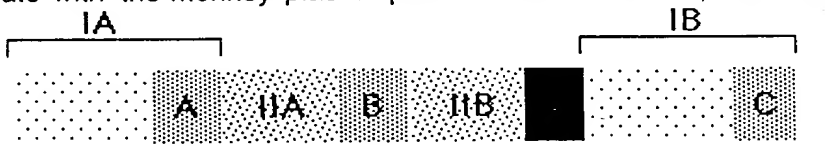
P39.5 *in vivo* and in amounts sufficiently high as to be immunogenic, since otherwise the anti-P39.5 antibody we had affinity purified would not have been elicited in the first place. In the blot shown in Fig. 4, the position of BmpA is indicated by its reaction with the anti-BmpA polyclonal antiserum (Fig. 4, lanes 2) and that of flagellin by the reaction of this molecule with Mab H9724 (Fig. 4, lanes 3). Lanes 1 of Fig. 4 depict the reactivity with serum obtained from a monkey infected with JD1. Lanes 5 are discussed in a section below. We had therefore identified a new antigen that is abundantly expressed *in vitro* by the *B. garinii* strain IP90 and *in vivo* by the *B. burgdorferi sensu stricto* strain JD1. The latter strain expresses this antigen very sparsely or not at all *in vitro*. This antigen, P39.5, appeared to be the target in the ADCK of IP90 spirochetes. We decided to clone it.

CLONING OF P39.5

A library of randomly-sheared total DNA from *B. garinii* IP90 was constructed in the λZAP II bacteriophage vector and screened with a pool of plasma collected from rhesus monkeys infected with the JD1 strain of *B. burgdorferi*. The plasma samples used for the pool were selected such that they contained antibody that recognized only P39.5, the putative flagellin and the unidentified higher molecular weight antigen. After several rounds of screening, eleven clones were rescued into the pBluescript phagemid, the recombinant plasmids were purified and used to transform cells of the SURE strain of *E. coli*. Several transformants were selected from each original clone, the presence of the insert was confirmed, and one such transformant from each clone was grown, induced for expression, lysed and analyzed by Western blot with the original plasma pool. The eleven cloned fragments hybridized to each other by dot-blot hybridization. One of the eleven clones (named 7-1) was selected for over-expression and purification on the basis of the strong reactivity of the expressed protein with the plasma antibodies. The 7-1 insert is 950-bp-long.

Figure 5: Western blot of lysates form IP90 spirochetes reacted with plasma from a monkey infected with JD1 spirochetes (lane 1), antibody from that plasma affinity purified with *B. garinii* clone 1 (lane 2) and clone 7-1 (lane 3)

The identity of the expressed protein was confirmed by showing that antibody from the original plasma sample that was affinity purified using the clone as immunoabsorbant reacted with P39.5 on a Western blot of *B. garinii* lysate. This is shown in Fig. 5. Reactivity of the IP90 lysate with the monkey plasma pool is shown in lane 1, with the antibody affinity purified with the recombinant 1-1 protein is shown in lane 2, and with the antibody affinity purified with the recombinant 7-1 protein is shown in lane 3.



p39.5

100 bp

Blocks	% Identity
IA vs. IB	70%
IIA vs. IIB	91%
A vs. B	84%
B vs. C	90%

Unique Region

Thus far we have been able to sequence 1190 bp, 950 of which were derived from clone 7-1 and 150 (5' to the 7-1 segment) from clone 14. The DNA fragment, which is depicted in the diagram on the left, encompasses a single open reading frame which encodes a deduced protein of 37.7 kDa. Its high alanine

EXHIBIT B

INFECTION AND IMMUNITY, Apr. 2000, p. 2349–2352 Vol. 68, No. 4
0019-9567/00/$04.00+0

Epitope Mapping of the Immunodominant Invariable Region of *Borrelia burgdorferi* VlsE in Three Host Species

FANG TING LIANG AND MARIO T. PHILIPP*

Department of Parasitology, Tulane Regional Primate Research Center, Tulane University Medical Center, Covington, Louisiana 70433

Received 3 November 1999/Returned for modification 1 December 1999/Accepted 21 December 1999

VlsE, the variable surface antigen of *Borrelia burgdorferi*, contains a 26-amino-acid-long immunodominant invariable region, IR_6. In the present study, three overlapping 14-mer peptides reproducing the sequence of IR_6 were used as peptide-based enzyme-linked immunosorbent assay antigens to map this invariable region in infected monkeys, mice, and human Lyme disease patients. Antibodies of the two primate species appeared to recognize IR_6 as a single antigenic determinant, while mouse antibodies recognized multiple epitopes within this region.

Borrelia burgdorferi sensu lato, the etiologic agent of Lyme disease (17), expresses a surface antigen, VlsE, which undergoes antigenic variation (21). Unlike other variable antigens, such as the variant surface glycoprotein of African trypanosomes (1, 5, 6, 20) and the variable major protein (Vmp) of *Borrelia hermsii* (3, 14, 18), which contain nonantigenic invariable portions, VlsE contains a 26-amino-acid-long invariable region (IR) which is immunodominant (11). This sequence (IR_6), which remains unchanged during antigenic variation (21), is highly conserved among strains and genospecies of *B. burgdorferi* sensu lato (11) and may thus play a critical role in maintaining the physiologic function of VlsE. Anti-IR_6 immunoglobulin G (IgG) antibody is readily detectable in both the early and late phases of *B. burgdorferi* infection in mice, monkeys, and humans (11, 12). However, since IR_6 is exposed at the surface of the VlsE molecule but not at the surface of the spirochete (11), anti-IR_6 antibody is likely not protective in vivo. On the other hand, the conservation and immunodominance of IR_6 indicates that anti-IR_6 antibody may have powerful diagnostic attributes. An enzyme-linked immunosorbent assay (ELISA) based on a peptide with the IR_6 sequence which is both sensitive and specific for the serodiagnosis of Lyme disease has been developed (12).

In this study, we attempted to map linear B-cell epitopes within IR_6 using sera from experimentally infected monkeys and mice and from humans clinically diagnosed with Lyme disease. A peptide-based ELISA was used.

The sequences of three overlapping 14-mer peptides were designed based on a consensus of IR_6 sequences from strains B31 (21) and 297 (10) of *B. burgdorferi* sensu stricto and IP90 of *Borrelia garinii* (11) (Fig. 1). The three 14-mers were named C_6N, C_6M, and C_6C. They were prepared using the fluorenylmethoxycarbonyl synthesis protocol (2). N-terminal conjugation to biotin was performed by the *N*-succinimidyl maleimide carboxylate method as per the instructions of the manufacturer (Molecular Probes, Eugene, Oreg.).

The peptide-based ELISA was performed as previously described (11). Briefly, 96-well ELISA plates were coated with streptavidin (Pierce Chemical Company, Rockford, Ill.) in coating buffer (0.1 M carbonate buffer, pH 9.2), followed by incubation with biotinylated peptides. Then antiserum and horseradish peroxidase-conjugated secondary antibody dilutions (goat anti-monkey IgG [γ chain specific; Kirkegaard & Perry Laboratories, Gaithersburg, Md.], anti-mouse IgG [heavy and light chain specific; Sigma Chemical Co., St. Louis, Mo.], or anti-human IgG [heavy and light chain specific; Pierce]) were applied. The antigen-antibody reaction was probed using a peroxidase substrate system (Kirkegaard & Perry), and optical density (OD) was measured at 450 nm. For competitive peptide-based ELISA, the biotinylated peptide C_6 was applied to the ELISA plate which had been coated with streptavidin. Competitive inhibition of anti-C_6 antibody was performed by adding 50 μl of 14-mer peptide(s) (C_6N, C_6M, C_6C, or a combination of two or three of the peptides), each between 0 and 5,000 ng per well. An equal volume of serum diluted 1:100 with blocking solution (phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, pH 7.4) was added to each well. The plate was shaken for 1 h. The remaining steps were performed as described for the peptide-based ELISA.

Ten serum samples derived from each host species were used to epitope map IR_6. Serum specimens were obtained from 10 rhesus monkeys (2- to 4-year-old *Macaca mulatta*), 9 of which had been inoculated by the bites of *Ixodes scapularis* nymphal ticks and 1 of which had been inoculated with needle and syringe. The ticks were themselves infected with either of the *B. burgdorferi* sensu stricto strains JD1 (15) and B31 (16). The needle-inoculated animal received JD1 organisms (15). Ten mice (6- to 8-week-old C3H/HeN mice; Jackson Laboratories, Bar Harbor, Maine) were infected either with *B. burgdorferi* sensu stricto strain Sh-2-82 (low passage number; a gift from Denee Thomas, University of Texas Health Science Center, San Antonio, Tex.) by subcutaneous needle inoculation with 10^8 spirochetes administered in 1 ml of BSK-H medium (Sigma) or with *B. burgdorferi* sensu stricto strain B31 by the bites of infected ticks. Serum samples were collected before and 4 to 6 weeks postinfection. Human serum samples were collected from 10 Lyme disease patients who had disease signs and symptoms that satisfied the Centers for Disease Control and Prevention clinical case definition (7). Five patients (A1 to A5) were diagnosed as having late Lyme arthritis, and five patients (N1 to N5) had late neuroborreliosis. Sera were kindly

* Corresponding author. Mailing address: Tulane Regional Primate Research Center, Tulane University Medical Center, 18703 Three Rivers Rd., Covington, LA 70433. Phone: (504) 871-6221. Fax: (504) 871-6390. E-mail: philipp@tpc.tulane.edu.

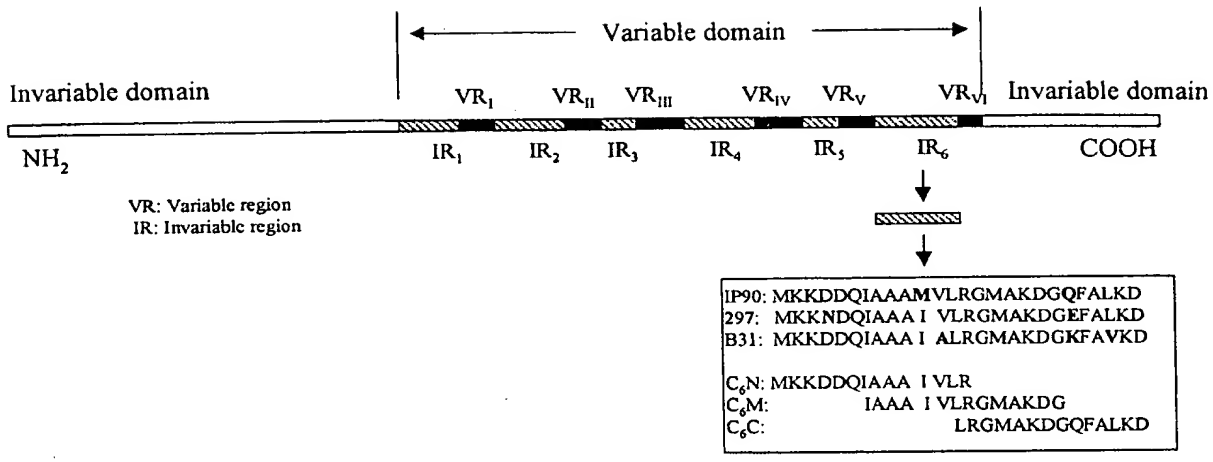


FIG. 1. Diagrammatic illustration of the VlsE structure. VlsE consists of two invariable domains at the amino and carboxyl termini and one variable domain at the center (21). The variable domain contains six variable regions, VR_1 to VR_{VI}, and six invariable regions, IR_1 to IR_6. The framed sequences show the IR_6 sequences from strains B31 (21) and 297 (10) of *B. burgdorferi* sensu stricto and IP90 of *B. garinii* (11). Bold letters indicate amino acids unique to each strain. The consensus sequences of the three overlapping peptides used in this study are depicted below (C_6N, C_6M, and C_6C). The C_6 sequence is based on that of IR_6 of IP90.

supplied by Allen Steere (New England Medical Center, Tufts University School of Medicine, Boston, Mass.).

Sera from 9 out of 10 infected monkeys did not contain detectable anti-14-mer peptide antibodies despite the presence of high levels of anti-C_6 antibody in virtually all of the specimens (Fig. 2). Only monkey J200 showed a significant reactivity with C_6C. These results indicate that this host species most likely recognizes the entire IR_6 segment as a single antigenic determinant.

Four out of 10 human serum samples showed reactivity with the overlapping peptide C_6M, but none contained detectable

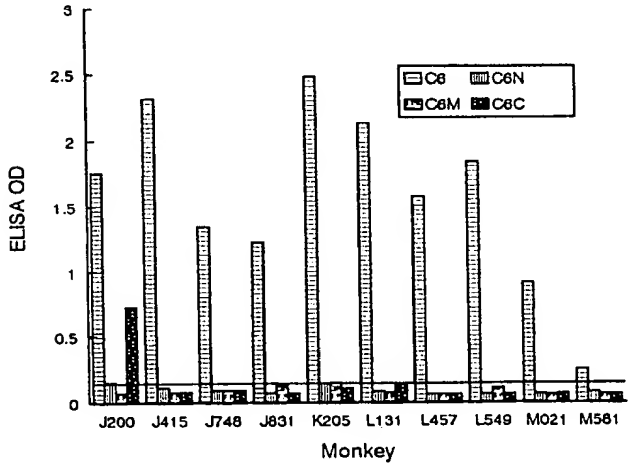


FIG. 2. Reactivities of overlapping peptides with monkey antibody. Serum samples were collected from monkeys at 4 to 6 weeks postinoculation. Animals were infected by tick inoculation either with the JD1 strain of *B. burgdorferi* (J200, J415, J831, K205, and L131) or with the B31 strain (L457, L549, M021, and M581). Animal J748 was needle inoculated with JD1 spirochetes. Levels of antibody to peptides were assessed using a peptide-based ELISA. Goat anti-monkey IgG–peroxidase conjugate was used as the secondary antibody. All serum samples were diluted 1:200. The cutoff line was based on the mean + 3 standard deviations of the OD values for 10 prebleeds that were reacted individually with each of the overlapping peptides and C_6.

antibodies to C_6N or C_6C (Fig. 3A). To determine what proportion of anti-C_6 antibody reactivity was contributed by antibody to C_6M, a competitive peptide-based ELISA was performed. The reactivity of specific antibody with C_6 in the four serum samples (A1, A2, N1, and N3) which reacted with C_6M was fully inhibited by increasing concentrations of C_6M (Fig. 3B). Even though serum N1 showed a very weak reactivity with C_6M in the peptide-based ELISA (Fig. 3A), a high concentration of this peptide was necessary to fully inhibit the reactivity with C_6 (Fig. 3B). The low reactivity with C_6M in the peptide-based ELISA but full inhibition in the competitive ELISA may have resulted from human antibodies with a lower affinity for C_6M than for C_6. C_6M may bind antibody as a partial epitope. No significant inhibition was observed when the peptides C_6N and C_6C were used in this assay (data not shown). Hence, the inhibition observed with the peptide C_6M should be considered specific. Taken together, our results suggest that humans, like monkeys, recognize IR_6 as a single epitope.

A different result was obtained when mouse sera were tested. High-level antibody responses to all three 14-mers were detected in several animals. Seven mice (mice 184, 219, 220, 224, 288, 289, and 290) had antibodies that reacted with the peptide C_6N. While serum from the first four animals reacted weakly, that of the latter three reacted as strongly as with the complete C_6 peptide (Fig. 4). Another set of three mice (mice 220, 288, and 290) had anti-C_6M antibodies, and five mice (mice 184, 191, 224, 289, and 290) possessed anti-C_6C antibodies (Fig. 4). Mouse 289 responded to both C_6N and C_6C but not to C_6M, indicating that this animal processed at least two independent epitopes at the amino and carboxyl termini of IR_6. Serum collected from mouse 288 reacted with both C_6N and C_6M, while serum from mouse 290 reacted with all three overlapping peptides. Competitive peptide-based ELISA revealed much more complicated antibody responses to IR_6 in mice than in monkeys and humans. Even the combination of the three overlapping peptides failed to fully inhibit the reactivity of serum 290 with the C_6 peptide (data not shown). The overlapping peptides C_6N and C_6M together also could not fully inhibit the reactivity of serum 288 with C_6, although C_6C did not react with this serum (data not shown). These results

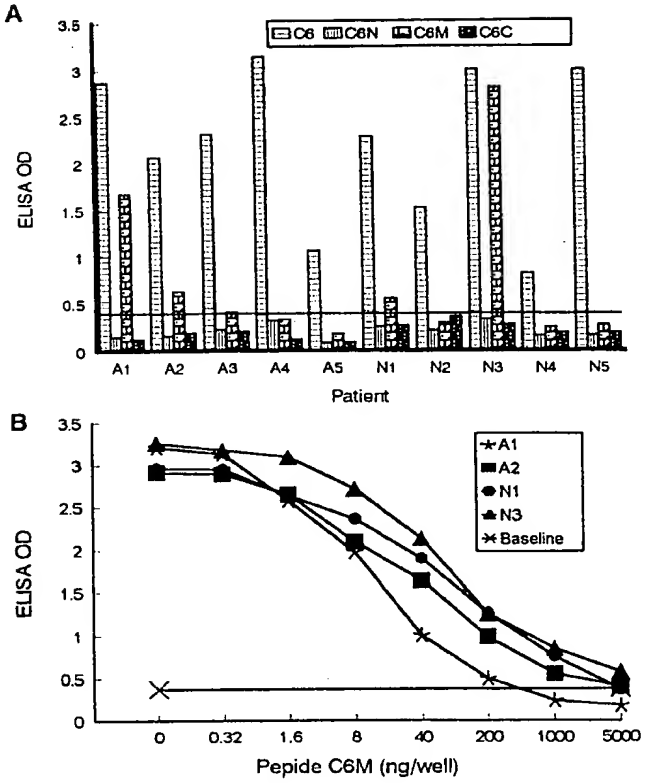


FIG. 3. Epitope mapping with human antibody. (A) Reactivities of overlapping peptides with human antibody. Ten sera were collected from patients with late Lyme arthritis (A1 to A5) or late neuroborreliosis (N1 to N5). Levels of antibody to peptides were assessed using a peptide-based ELISA. Goat anti-human IgG–peroxidase conjugate was used as the secondary antibody. All serum samples were diluted 1:200. The cutoff line (baseline) value is the mean + 3 standard deviations of OD values for 10 human serum specimens collected from hospitalized patients in Louisiana, where Lyme disease is not endemic. For this purpose, the three overlapping peptides and C_6 were individually used as an ELISA antigen. (B) Inhibition of human antibody reactivity with C_6 by C_6M. Amounts of C_6M ranging from 0 to 5,000 ng per well were added to the C_6-bound ELISA plate. Human serum diluted 1:100 was applied. The remaining steps were performed as described for panel A.

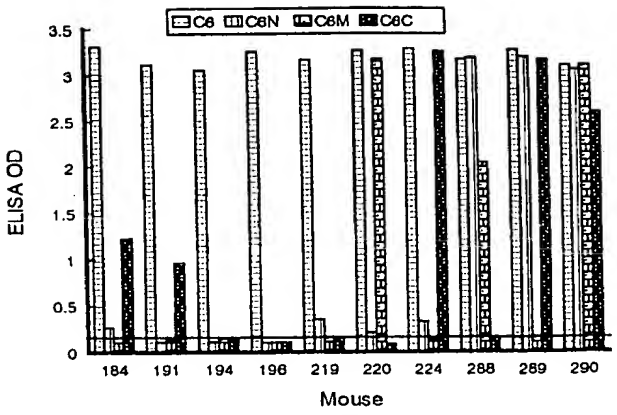


FIG. 4. Reactivities of overlapping peptides with mouse antibody. Serum samples were collected from mice at 4 to 6 weeks postinoculation. Animals were infected with either *B. burgdorferi* sensu stricto strain B31 (mice 184, 191, 194, and 196) by tick inoculation or Sh-2-82 (mice 219, 220, 224, 288, 289, and 290) by needle inoculation. Levels of antibody to peptides were assessed using a peptide-based ELISA. Goat anti-mouse IgG–peroxidase conjugate was used as the secondary antibody. Sera were diluted 1:200. The cutoff line was based on the mean + 3 standard deviations of OD values for all of the 10 prebleeds reacted individually with each overlapping peptide and C_6.

suggest that mice are able to recognize multiple epitopes within IR_6.

Although we cannot fully exclude the possibility that the antibody response to 14-mer portions of IR_6 was exacerbated in mice because of the mode of inoculation (needle versus tick inoculation) or the strain of spirochete used (B31 versus Sh-2-82), we do not believe that these factors may fully explain the difference we observed in the responses to these sequences in the host species studied. The mode of inoculation may influence the antibody response because a higher dose of spirochetes is likely received by needle- than by tick-inoculated animals, and immune-suppressive substances are inoculated by the tick concomitantly with spirochetes (19). All of the six needle-inoculated mice that received the Sh-2-82 strain responded to one or more of the 14-mers, yet half of the four tick-inoculated mice also responded to C_6C and one responded weakly to C_6N. Monkey J748, which was needle inoculated, did not respond to any of the 14-mer peptides. As to spirochete strain differences, it is possible that spirochetes of the Sh-2-82 strain express a higher level of VlsE than that expressed by B31 organisms in vivo and may thus facilitate antipeptide responses. This we cannot exclude, but the fact that none of the four tick-inoculated monkeys that received the B31 strain responded to 14-mers whereas two of the four B31 tick-inoculated mice did underscores host species difference as the major reason for the differential responsiveness to fragments of IR_6 that we found.

Most B-cell epitopes of native protein antigens are discontinuous in that they encompass regions which are separate from each other in the primary sequence of the polypeptide chain but are brought together on the native molecule by protein folding (4). Our results suggest that IR_6 may function as a single epitope, probably nonlinear in view of its length, in primate species, human or nonhuman, but that IR_6 may behave as a collection of multiple overlapping linear epitopes in mice. Analysis of the secondary structure of IR_6 by either the Chou-Fasman (8) or the Robson-Garnier (9) algorithms (MacVector 4.1; Eastman Chemical Co., New Haven, Conn.) predicted an α-helix comprised of an 11-mer sequence [AA(I or M)(A or V)LRGMAKD] regardless of whether the B31, 297, or IP90 IR_6 sequence was used in the calculation. The 11-amino-acid segment is located at the center of IR_6 and is fully included in the C_6M peptide (Fig. 1). In humans, this helix may serve as the epitope core, for when C_6M was used as a competitor, it was able to fully inhibit the reactivity of human serum antibody with C_6, albeit at a high concentration of the peptide (Fig. 3). The sequences immediately flanking the α-helix probably contribute to the binding affinity either by allowing the α-helix to "grow" or by contributing some tertiary structure to it, since human antibody yielded a higher ELISA OD with C_6 than with C_6M. The remarkable antigenicity of IR_6 in several host species argues against the presence of additional, discontinuous regions of VlsE as contributors to the native antigenicity of IR_6. In fact, all experimentally infected mice and monkeys we have tested thus far (10 mice and 10 monkeys), most human patients with early *B. burgdorferi* infections (117 of 138), and all patients with a late, clinically well defined infection (we tested 59) had detectable anti-IR_6 antibodies (11,

12). Thus, while the totality of IR_6 appears to be required to express the full antigenicity of this region, it is unlikely that other VlsE portions are required as well. Our studies have uncovered striking differences in the antibody responses to the invariable regions of VlsE in different host species. We had noted previously that mouse antibodies recognized IR_2, IR_4, and IR_6 but that monkey and human antibodies reacted essentially only with IR_6 (13). The present study revealed that primate host species recognize IR_6 in toto but that mice appear to detect multiple linear and overlapping epitopes within this region. If VlsE plays a role in the host-microbe interplay of *B. burgdorferi*, these differences in B-cell antigen recognition patterns could contribute to explain, once that role has become clear, differences in the natural histories of murine and primate Lyme disease. At a more general level, this finding illustrates that B-cell epitope mapping is host dependent.

This work was supported by grants AI35027 and RR00164 from the National Institutes of Health and by a grant from SmithKline Beecham Biologicals.

REFERENCES

1. **Barbet, A. F., and T. C. McGuire.** 1978. Crossreacting determinants in variant-specific surface antigens of African trypanosomes. Proc. Natl. Acad. Sci. USA **75**:1989–1993.
2. **Barony, G., and R. B. Merrifield.** 1980. The peptides: analysis, synthesis, & biology, p. 3–285. Academic Press, New York, N.Y.
3. **Barstad, P. A., J. E. Coligan, M. G. Raum, and A. G. Barbour.** 1985. Variable major proteins of *Borrelia hermsii*. Epitope mapping and partial sequence analysis of CNBr peptides. J. Exp. Med. **161**:1302–1314.
4. **Benjamin, D. C.** 1995. B-cell epitopes: fact and fiction, p. 95–108. *In* L. M. Aledort et al. (ed.), Inhibitors to coagulation factors. Plenum Press, New York, N.Y.
5. **Borst, P.** 1991. Molecular genetics of antigenic variation. Immunol. Today **12**:A29–A33.
6. **Borst, P., and G. A. M. Cross.** 1982. Molecular basis for trypanosome antigenic variation. Cell **29**:291–303.
7. **Centers for Disease Control and Prevention.** 1997. Case definition for infectious conditions under public health surveillance. Morbid. Mortal. Weekly Rep. **46**:20.
8. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformations. Annu. Rev. Biochem. **47**:251–276.
9. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
10. **Kawabata, H., F. Myouga, Y. Inagaki, N. Murai, and H. Watanabe.** 1998. Genetic and immunological analyses of Vls (VMP-like sequences) of *Borrelia burgdorferi*. Microb. Pathog. **24**:155–166.
11. **Liang, F. T., A. L. Alvarez, Y. Gu, J. M. Nowling, R. Ramamoorthy, and M. T. Philipp.** 1999. An immunodominant conserved region within the variable domain of VlsE, the variable surface antigen of *Borrelia burgdorferi*. J. Immunol. **163**:5566–5573.
12. **Liang, F. T., A. C. Steere, A. R. Marques, B. J. B. Johnson, J. N. Miller, and M. T. Philipp.** 1999. Sensitive and specific serodiagnosis of Lyme disease by ELISA with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. J. Clin. Microbiol. **37**:3990–3996.
13. **Liang, F. T., and M. T. Philipp.** 1999. Analysis of antibody response to invariable regions of VlsE, the variable surface antigen of *Borrelia burgdorferi*. Infect. Immun. **67**:6702–6706.
14. **Meier, J. T., M. I. Simon, and A. G. Barbour.** 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia*. Cell **41**:403–409.
15. **Philipp, M. T., M. K. Aydintug, R. P. Bohm, Jr., F. B. Cogswell, V. A. Dennis, H. N. Lanners, R. C. Lowrie, Jr., E. D. Roberts, M. D. Conway, M. Karaçorlu, G. A. Peyman, D. J. Gubler, B. J. B. Johnson, J. Piesman, and Y. Gu.** 1993. Early and early disseminated phases of Lyme disease in the rhesus monkey: a model for infection in humans. Infect. Immun. **61**:3047–3059.
16. **Philipp, M. T., Y. Lobet, R. P. Bohm, Jr., E. D. Roberts, V. A. Dennis, Y. Gu, R. C. Lowrie, Jr., P. Desmons, P. H. Duray, J. England, P. Hauser, J. Piesman, and K. Xu.** 1997. The outer surface protein A (OspA) vaccine against Lyme disease: efficacy in the rhesus monkey. Vaccine **15**:1872–1884.
17. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321**:586–596.
18. **Stoenner, H. G., T. Dodd, and C. Larsen.** 1982. Antigenic variation of *Borrelia hermsii*. J. Exp. Med. **156**:1297–1311.
19. **Urioste, S., L. R. Hall, S. R. Telford III, and R. G. Titus.** 1994. Saliva of the Lyme disease vector, *Ixodes dammini*, blocks cell activation by a nonprostaglandin E2-dependent mechanism. J. Exp. Med. **180**:1077–1085.
20. **Van der Ploeg, L. H. T., K. Gottesdiener, and M. G. S. Lee.** 1992. Antigenic variation in African trypanosomes. Trends Genet. **8**:452–457.
21. **Zhang, J. R., J. M. Hardham, A. G. Barbour, and S. J. Norris.** 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell **89**:275–285.

Editor: R. N. Moore